“Breaking up is hard to do”: the formation and resolution of sister chromatid intertwines

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The formation and resolution of sister chromatid intertwines

Jonathan Baxter[1]

[1]Genome Damage and Stability Centre,

Science Park Road,

University of Sussex,

Falmer, Brighton,

East Sussex, BN1 9RQ, UK.

(Jon.Baxter@sussex.ac.uk)

Abstract

The absolute necessity to resolve every intertwine between the two strands of the DNA double helix provides a massive challenge to the cellular processes that duplicate and segregate chromosomes. Although the overwhelming majority of intertwines between the parental DNA strands are resolved during DNA replication, there are numerous chromosomal contexts where some intertwining is maintained into mitosis. These mitotic sister chromatid intertwines (SCI) can be found as short regions of unreplicated DNA, fully replicated and intertwined sister chromatids - commonly referred to as DNA catenation - and as sister chromatid linkages generated by homologous recombination associated processes. Several overlapping mechanisms, including intra-chromosomal compaction, topoisomerase action and Holliday junction resolvases ensure all SCIs are removed before they can prevent normal chromosome segregation.

Here, I discuss why some DNA intertwines persist into mitosis and review our current knowledge of the SCI resolution mechanisms that are employed in both prokaryotes and eukaryotes, including how deregulating SCI formation during DNA replication or disrupting the resolution processes may contribute to aneuploidy in cancer.

Main text

The separation of condensed mitotic chromosomes is an iconic process in cell biology, first described over a century ago [1]. Studies of separating mitotic chromosomes found that occasionally chromosomes could not be properly separated, causing chromosomal bridges between the two segregating masses. This begged the question of why the segregating chromosomes could not be separated. What was the nature of the connections that prevented sister chromosomes from being pulled apart?

The anaphase bridging of chromosomes was initially attributed to the rare formation of "dicentric" chromosomes, by the classic "break-fusion-bridge cycle" proposed by Barbera McClintock [2]. This would lead to a single chromosome being pulled to both poles of a dividing cell. However, it has now become clear that interfering with the processes that remove the intertwines between the two strands of the DNA double helix during chromosome duplication also leads to chromosome bridging in anaphase. In this review, I will explore the nature of the residual DNA linkages that exist between sister-chromatids that can prevent their segregation in mitosis, including the origins of their formation and the mechanisms that resolve them. These mitotic connections, often referred to as sister chromatid intertwines (SCI), come in three different types (see graphical abstract): Short regions of unreplicated DNA, where the single stranded intertwines of the parental, template DNA duplex maintain a connection between the otherwise fully replicated chromosomes. DNA catenation, where the removal of the template DNA intertwines is uncoupled from replication, leading to a double stranded DNA intertwines between the sister chromatids. Also sister chromatid junctions (SCJ), that are formed when a strand from one of the newly replicated sister chromatids forms a heteroduplex with the complementary strand of the

other sister chromatid. The formation and resolution of each type of SCI will be discussed in turn. However, I will first discuss how all the DNA intertwines that do not form SCI are resolved during DNA replication, along with a brief description of the enzymes responsible for resolving these DNA linkages, the topoisomerases.

The nature of DNA intertwines and topoisomerase action

It is now over 60 years since the double helical structure of DNA was elucidated by Watson and Crick [3,4]. The intertwining of the two DNA strands every 10.4 base pairs in this structure immediately presented (and continues to pose) huge mechanistic challenges. For the DNA polymer to be the medium of genetic inheritance every linkage between the two strands has to be removed to allow the duplication and segregation of the genetic code to the daughter cells. Despite recognising this fundamental problem, with characteristic insouciance the two authors stated that “although it is difficult at the moment to see how these processes occur without everything getting tangled, we do not feel this objection will be insuperable” [4].

However, when the double helical structure of DNA was revealed, there was simply no mechanism known as to how the cell could resolve the almost countless intertwines that appeared to exist between the two strands of nucleic acid. It was recognised early on that an efficient mechanism of resolving the connections would be to introduce transitory breaks in the strands [5] but it was another thirty years before cellular enzymes capable of such a function were identified.

The discovery of abundant topoisomerase enzymes in all living cells provided a mechanism for removing the intertwines within DNA (discussed in [6]). Topoisomerases directly cleave DNA strands, allowing for changes in the intertwining of the strands, before then re-ligating

the cleaved strands together. In this way they can resolve all the connections between the two intertwined parental DNA strands.

The exact mechanisms by which topoisomerases achieve intertwining changes varies dependent on the particular enzyme and the context of its action (reviewed in detail by Vos *et al*. [7]). Briefly, topoisomerases are grouped into two types; type I enzymes that cleave only one strand of the DNA and type II enzymes that cleave both strands of the DNA duplex. Type IA enzymes cleave one strand of DNA and then pass the second strand through it by a "strand passage" mechanism. Type IB enzymes change the intertwining of the DNA duplex by nicking one strand of the DNA duplex and allowing the strands to rotate relative to one another. Type II topoisomerases employ a strand passage mechanism similar to type IA enzymes. However, type II enzymes cleave both strands of DNA on one stretch of DNA (the gate or G segment) before passing a second intact double stranded DNA segment (the transfer or T segment) through the break.

Topoisomerases generally target DNA that is topologically stressed, i.e. it is either overwound or underwound compared to the ideal winding frequency of B-form DNA, one intertwine every 10.4 base pairs. Usually DNA that is topologically stressed becomes supercoiled. Supercoiling occurs because it is energetically favourable for DNA to maintain B-form, so any overwinding or underwinding introduced, leads to coiling of the fibre around itself. Supercoiling due to overwinding is called positive supercoiling while supercoiling due to underwinding is called negative supercoiling (Figure 1A). (For a more complete explanation of intertwining/linking number changes in DNA and supercoiling please see Postow et al. [8] or Schvartzman and Stasiak [9]). Both type IB and type II topoisomerases can relax both positive and negative supercoiled DNA (Figure 1A,C), causing a change in the number of intertwines between the strands (relaxing positive supercoiling decreases the number of intertwines between the strands while relaxing negative supercoiling increases the number of intertwines between the strands). Since the strand passage reaction of type

IA enzymes requires single stranded DNA, these preferentially act on negatively supercoiled/ unwound DNA (Figure 1B). Due to their ability to cleave both strands of DNA, the type II enzymes can also act inter-molecularly, to remove (or add) catenated intertwines (Figure 1D) as well as acting on DNA supercoiling (Figure 1C).

Removing DNA linkages during DNA replication through the relaxation of positive supercoiling

Since most topoisomerase will not change the number of intertwines within the relaxed DNA duplex, other mechanisms have to introduce local changes in winding during chromosome duplication to target topoisomerase action to remove the intertwines. During DNA replication this is achieved by co-ordinating the action of the replicative helicase and either a type IB or a type II topoisomerase (Figure 2A (i)-(iv)). In the course of separating the two strands of the double helix the replicative helicase breaks base pairing between the strands and displaces the associated intertwines into the region ahead of the fork. Since helicase action forces the strands apart without removing the intertwining, the effect of this is to generate overwinding or positive supercoiling into the region ahead of the fork (Figure 2A(ii)). If allowed to build up this positive supercoiling stress would rapidly stall DNA replication. However, both type II and type IB topoisomerases rapidly relax the stress, thus removing the intertwines between the parental strands that were displaced by helicase action (Figure 2A (iii) and 2A (iv) (reviewed by Wang [10])). The vast majority of the links between the two strands of the double helix appear to be removed during DNA replication in this fashion.

Single stranded SCI – unreplicated DNA

Clearly, failure to resolve all the intertwines in the template DNA helix will lead to problems in separating the partially replicated chromosomes in mitosis and the appearance of chromosome bridges in anaphase. Inhibiting DNA replication with pharmacological agents

triggers the formation of anaphase bridges [11-13]. So the data suggest that a failure to fully replicate DNA before mitosis leads to single stranded SCI between mitotic chromosomes. How such structures would be resolved *in vivo* is unclear, although *in vitro* similar single stranded intertwined structures can be resolved by the conserved RecQ helicase and type IA topoisomerase Topoisomerase III [14,15] . It is thought that the action of the RecQ helicase instigates sufficient unwinding for the single strand passage activity of topoisomerase III to resolve the single stranded intertwines (Figure 2B). In addition the SCI generated by inhibition of DNA replication in mammalian cells are often bound at their ends by FANCD2 and along their length by the mammalian RecQ helicase BLM [12] (see section below on cytological visualisation of intertwines) suggesting that the Fanconi anaemia pathway and RecQ helicase action may both be involved in their resolution. Although single stranded SCI could be a primary source of anaphase bridges in cancer cells experiencing replicative stress (see section on SCI and cancer below), their physiological relevance in unperturbed cells is unknown. In contrast another type of SCI, generated in every S phase, has been shown to be a physiological intermediate of genome duplication. This type of SCI is generated from the intertwining of newly replicated DNA strands, and is generally referred to as DNA catenation.

DNA catenation

Early studies of bacterial and viral DNA replication demonstrated that the action of topoisomerases ahead of the fork could not resolve every DNA intertwine within a replicon. These experiments on plasmid DNA showed that at the later stages of replication, the parental strands between the converging replication forks were not resolved ahead of the helicase (Figure 3). Instead they were converted into double stranded DNA intertwines, referred to as DNA catenanes on the circular plasmids (reviewed in detail by Postow et al. [8]). Technically, a DNA catenane can only be formed between linked circular DNA plasmids, but most of the studies on the generation of double stranded DNA SCI have used

circular DNA substrates and therefore refer to them as DNA catenanes. To maintain consistency I will refer to double stranded DNA SCI on both circular and linear chromosome as DNA catenanes in this review to help distinguish them from other types of SCI discussed.

The explanation for why DNA catenation occurs in cells was first proposed by Champoux and Been [16]. They recognised that the overwinding, superhelical tension generated ahead of the fork by helicase action could be diffused into the region behind the replication fork if the whole fork could rotate relative to the unreplicated DNA (Figure 2A (v)). As a consequence of fork rotation the topological stress ahead of the fork is relaxed at the expense of generating DNA catenation behind the fork. So two pathways can be utilised to resolve the single stranded intertwines in the parental, template DNA – one that transforms the single stranded intertwines ahead of the fork into double stranded intertwines behind the fork by fork rotation (which will require subsequent type II topoisomerase decatenation) and one that resolves the template DNA single stranded intertwines directly, in the topologically stressed region ahead of the fork (type IB or type II topoisomerase action on the positive supercoiling generated by replisome helicase action) (Figure 2A).

The generation of DNA catenanes behind the replisome by fork rotation is most commonly associated with the termination of DNA replication (Figure 3). It was first shown in SV40 DNA replication that catenated sister chromatids were a normal late replication intermediate of the virus [17,18]. Using culture conditions to block decatenation activity Sundin and Varshavsky showed that on average 10 intertwines were generated during replication of the 5.2 kb circular chromosome. These studies indicated that fork rotation was not generally utilized to remove template DNA intertwines - on average it is utilised to unwind only 10 of the @ 500 intertwines between the parental strands of SV40, but it become very important for unwinding where the two converging replisomes came together at the termination of DNA replication.

Topoisomerase action and DNA catenation

Following on from these ground-breaking studies most of the subsequent work on the frequency of fork rotation and the generation of DNA catenation has been carried out in *E. coli*. In this organism, the availability of both *in vivo* genetics and a fully reconstituted *in vitro* DNA replication system have provided numerous insights into when DNA catenation occurs during DNA replication. In *E. coli* two different type II topoisomerases, DNA gyrase and topo IV, normally resolve all of the DNA intertwines during replication. DNA gyrase is focused on unwinding the DNA ahead of the replication fork. This is due to its unique C-terminal domain that promotes an intra-chromosomal strand passage activity [19]. Therefore the enzyme can efficiently remove intertwines in the parental template DNA by either relaxing the supercoiling generated by the replisome or introducing negative supercoiling (unwinding) into the DNA. However, DNA gyrase cannot efficiently promote the inter-chromosomal strand passage activity required for decatenation under physiological conditions [20]. In contrast, topo IV can operate both ahead of and behind the fork; efficiently removing template strand intertwines ahead of the fork by relaxing replisome generated positive supercoiling, or removing the double stranded DNA catenanes produced in the newly replicated DNA by fork rotation [20-22]. *In vitro* both DNA gyrase and topo IV can efficiently support the elongation phase of DNA replication [22,23]. However, only topo IV can support rapid replication during the late stages of DNA replication when DNA catenation occurs and is resolved [22]. This suggests that fork rotation and the subsequent resolution of DNA catenation is the primary pathway to resolving the linkages on the template DNA in these late stages. Consistent with this, topo IV is required for genome segregation in *E. coli* [24]. Therefore several characteristics of DNA catenation are shared between the SV40 and *E. coli* systems; DNA catenanes are a normal intermediate of replication, and type II topoisomerase decatenation activity is essential in cells to resolve the catenated replicated chromosomes. In both systems it appears that the relaxation of replication induced topological stress by fork rotation and catenation occurs relatively infrequently compared to

topoisomerase action on positive supercoiling ahead of the fork - in *E. coli* genetic ablation of topo IV activity leads to the accumulation of between 2-16 DNA catenanes on a 4.3 kb plasmid [20]. Also in both cases DNA catenation appears to be primarily associated with converging replication forks.

In eukaryotes, two topoisomerases are involved in unlinking during DNA replication, the type IB topoisomerase I (Top1 in yeast) and the type II topoisomerase II (Top2 in yeast). *In vitro* both Top1 and Top2 can relax both negative and positive supercoiling, although eukaryotic topoisomerase II enzymes often have a preference for positive supercoiling [25-27]. Genetic ablation of either Top1 or Top2 alone in yeast does not alter the kinetics of DNA replication, however ablation of both topoisomerases leads to an apparently immediate cessation of DNA replication [28,29]. These data indicate that either topoisomerase is sufficient for resolving intertwines during bulk DNA replication, and that no other topoisomerase activity is available in the cell to substitute for the loss of both. Since both topoisomerases are capable of relaxing positive supercoils, they can both act ahead of the replisome, unlinking the parental template DNA through relaxation of the topological stress generated by helicase action. As expected, only Top2 can decatenate the DNA catenanes generated by rotation of the replisome to relax stress ahead of the fork [30,31]. Since conditional genetic ablation of Top2 prevents decatenation, the number of DNA catenanes formed on plasmid replicons during DNA replication in yeast cells can be directly assayed. These assays have shown that fork rotation on yeast episomal plasmids is relatively infrequent and generally limited to the regions around converging forks in a similar manner to SV40 or *E. coli* [31,32].

The current model of why replication fork rotation occurs primarily in the late stages of DNA replication is framed in terms of enzyme accessibility [8]. This model posits that in the early stages of DNA replication there are no impediments to DNA topoisomerases acting ahead of the replication forks. However as forks converge, the region of unreplicated DNA that topoisomerases can act on becomes progressively more limited until the convergence of the

large replisome structures eventually prevents topoisomerase action between them (Figure 3). As the inhibition of acting ahead of the fork increases so the relative energetics of fork rotation and DNA catenation become more favourable.

The influence of replisome structure on DNA catenation

A crucial unknown factor in this model is the *in vivo* resistance of the replisome holo-complex to rotation (Figure 4). The level of resistance to rotation of the replisome at the fork would presumably regulate the equilibrium between topoisomerase action ahead of the fork versus fork rotation and action behind it. When de-proteinized, topological stress ahead of the fork can easily diffuse into replicated regions by fork rotation, generating DNA catenanes [33]. However the expected resistance to fork rotation when a complete replisome holo-complex is localised to the fork is unquantified. Clearly a functional form of the replisome can rotate as evidenced by the general formation of catenated plasmids following replication. Also *in vitro* experiments in *E. coli* have shown that a functional replisome can efficiently replicate a plasmid template utilizing only fork rotation and action behind the fork of a type IA enzyme (which can only act within the single stranded regions behind the fork) to resolve all the DNA linkages [34]. However comparing different studies suggests that the resistance to rotation may be significantly higher *in vivo* compared to *in vitro*. *In vitro* topo IV can support elongation rates that are comparable to those of gyrase [23]. However, the elongation rate of DNA replication *in vivo* drops to a third of wild type when DNA gyrase is specifically inhibited [35] whereas loss of topo IV activity has little influence on replication rates [36]. Therefore, *in vivo,* topo IV does not have an equivalent role to gyrase during elongation of DNA replication. The *in vivo* replisome holo-complex is likely to require more associated activities – and therefore more proteins – at any given time to cope with the numerous barriers to replication that are likely to be encountered during *in vivo* DNA replication [37]. Increasing the size of the minimal holo-enzyme probably increases the resistance to rotation of the complex. This increase of *in vivo* rotation resistance is likely to be even greater in

eukaryotes than in prokaryotes. More proteins appear to be associated with the eukaryotic replisome and the replisome has several additional roles such as nascent chromatin deposition and cohesion [38,39]. The association of such additional non-core factors would presumably also increase the rotational drag of the holo-complex (Figure 4). In addition, if active replisomes are physically connected within replication factories in the nucleus [40,41], this would be predicted to generate further structural rigidity that would impede fork rotation.

Frequency of fork rotation and catenane formation by elongating replication forks

So *in vivo* it is likely that the equilibrium between topoisomerase action ahead of the fork and fork rotation (and the consequent generation of catenated double stranded SCI) is firmly in favour of topoisomerase action ahead of the fork, outside the specialised context of termination of DNA replication. However, there is direct evidence that some fork rotation does take place during elongation in *E. coli* and indirect evidence that fork rotation occurs during elongation in eukaryotic systems. In *E. coli*, cytological marking of DNA loci with fluorescent proteins has shown that a number of newly replicated loci appear “cohesed” for a short period following replication [36]. The time of cohesion following replication is directly related to topo IV dosage, with decreased dosage increasing the time of cohesion and increased dosage reducing it [36], arguing that the cohesion was maintained by DNA catenations formed by fork rotation and resolved by topo IV action. Therefore it seems reasonable to assume that a small amount of fork rotation and DNA catenation occurs during elongation to maintain transient cohesion behind the fork.

In eukaryotes it is far more difficult to discern whether DNA catenations are formed during both elongation and termination or only at termination, since there are many termination events during eukaryotic DNA replication, stochastically spread throughout the genome [42,43]. Nevertheless there are a number of observations that suggest that DNA catenation is enriched in certain chromosomal regions above a level that could be explained solely by

termination events. In human cells, topoisomerase II alpha is substantially enriched compared to topoisomerase I in inactive areas of the genome, which tend to be heterochromatic [44] including the centromeric heterochromatin [45]. Inhibition of topoisomerase II in mitosis leads to a substantial increase in ultrafine anaphase bridges originating from centromeric regions [46], arguing that these regions are disproportionally enriched for DNA catenation (see below). In yeast the rDNA region, which is primarily composed of silent rDNA repeats, appears to be catenated to a more significant extent than would be expected by termination alone [47].

At present, we do not understand why the frequency of rotation and DNA catenation would increase in regions such as the centromeric heterochromatin. However, there does appear to be one situation where fork rotation is significantly increased on plasmid substrates. A study in budding yeast has shown that overexpression of inactive topoisomerase II during DNA replication substantially increases the frequency of fork rotation and DNA catenation on a plasmid substrate [31]. Expression of the inactive topoisomerase increased the average number of fork rotations from 12 (when all topoisomerase protein has being depleted) to around 30 ([31] and unpublished data). In both cases all decatenation activity had been inhibited, but the presence of the inactive topoisomerase in the cell (which can bind to but not metabolise DNA) some how triggered increased fork rotation. A similar phenomena is observed in other organisms; in *E. coli* genetic ablation of topo IV leads to 2-16 catenations being stabilised on a plasmid [20] whereas inactivation of topo IV with quinolone – which crosslinks the topoisomerase to the DNA - leads to 2- 68 DNA catenations on the same plasmid [48]. Interestingly, quantitation of fork rotation during replication of a small plasmid in *Xenopus* extracts using the drug ICRF-193 to inactivate topoisomerase II demonstrated that >40 catenanes are formed during replication of the plasmid [49]. This number of catenanes is far in excess of the number produced in other systems. This could suggest that the resistance to replisome rotation is significantly lower in *Xenopus* than in other systems, although there is currently no evidence to suggest this is the case. Or it could indicate that

the topoisomerase-DNA complex produced by ICRF-193 increases the frequency of fork rotation during DNA replication. One mechanism for increased fork rotation following topoisomerase inhibition is that the DNA bound inactive topoisomerase prevents the binding of other active topoisomerases ahead of the fork. Inhibition of topoisomerase action ahead of the fork would directly favour fork rotation increasing the extent of catenation on replicated plasmids. The inhibition of topological relaxation ahead of the fork by competition for the substrate DNA, that leads to fork rotation and DNA catenation, is analogous to the model of why fork rotation occurs at termination – in both cases the energetics of acting ahead of the fork become relatively far more costly. This in turn makes the energetic cost of fork rotation more favourable, resulting in more catenated sister chromatids. Potentially this mechanism could be extended to other DNA contexts where it is plausible that topoisomerase action ahead of the fork may be inhibited. Certainly the densely packaged chromatin environment of heterochromatin could inhibit the binding frequencies of topoisomerase ahead of the fork, potentially leading to increased fork rotation in these regions.

In summary DNA catenation appears to be a universal intermediate of DNA replication. The frequency of formation appears to be context dependent, with situations that hinder topoisomerase action ahead of the fork, such as the convergence of replisomes at termination, leading to significantly increased frequencies of DNA catenation. Whatever the extent of DNA catenation produced during DNA replication it is essential that every catenane generated between the sister chromatids is resolved to allow faithful segregation of the chromosomes into the daughter cells.

The complete resolution of DNA catenations

In order for all of the duplicated chromosomes to be faithfully segregated to daughter cells it is essential that all DNA catenanes (and indeed all other types of SCI) between the sister chromatids are resolved before cell division. The complete resolution of DNA catenation is

the essential activity of type II topoisomerases in both bacteria and eukaryotes [20,30]. However, the strand passage activity of type II topoisomerases is capable of generating a double strand break in any stretch of DNA and then passing any proximal stretch of DNA through the gap. The topoisomerase enzyme itself is “blind” to the wider context of this strand passage reaction. In order to facilitate the segregation of chromosomes the type II topoisomerase activity has to somehow be focused on strand passage reactions that remove catenated linkages as opposed to the other possible outcomes of the reaction. Failure to do this could lead to the strand passage activity of the topoisomerase introducing linkages into the sister chromatids rather than removing them (Figure 1D). In the crowded post-replicative DNA environment of a cell, where the replicated chromosomes are intermingled and the two sister chromatids are closely juxtaposed by proteinaceous cohesin complexes, this is a potentially serious problem. Indeed, full cohesion of DNA plasmids by cohesin ensures maintenance of higher level of DNA catenation between them than is observed following cohesin removal [50]. Therefore, there are several overlapping mechanisms that ensure the full decatenation of sister chromatids *in vivo* by type II topoisomerases.

The decatenating type II enzymes appear to have an intrinsic ability to decatenate DNA *in vitro* below the expected thermodynamic equilibrium, presumably supported by ATP hydrolysis [51]. Mechanistically, this has been proposed to occur through bending of the segment of DNA that is broken, thus biasing selection of the DNA segment to be transferred through the break to catenated rather than uncatenated DNA molecules [52]. However, the quantitated shift in equilibrium dynamics is not sufficient to fully decatenate sister chromatids (discussed by Stuchinskaya *et al*. [53]).

A universally important mechanism of driving decatenation is intra-chromosomal compaction. Note the crucial aspect of this mechanism is that the compaction is self-directed onto each individual chromosome and not general. General condensation of a mixture of

chromosomes is liable to produce a more highly catenated state by increasing the proximity of distinct chromosomes to each other and thus promoting their catenation by type II topoisomerases, as demonstrated *in vitro* [54]. In contrast, intra-chromosomal compaction forces each chromosome into its own, separate, volume. Any connection between two different chromosomes will be excluded from the self-compacting volume with the energetics of resolution of the connection by type II topoisomerases becoming increasingly favourable as the volume becomes more compact [55] (Figure 5). Note that this intra-chromosomal compaction will drive the resolution not only of double stranded DNA intertwines/catenation but also the resolution of all other types of resolvable connections and intertwines between the sister chromatids. One mechanism of active self-compaction found in both prokaryotes and eukaryotes is the introduction of DNA supercoiling into the newly replicated sister-chromatids. Introducing supercoiling into a DNA polymer forces it to coil in on itself (Figure 5A), the same way an old fashioned telephone wire will compact itself if overwound. This allows action at any point on a DNA polymer to produce self-directed compaction over a potentially long distance. The details of this mechanism have been most clearly elucidated in bacteria where the introduction of negative supercoils into the newly replicated DNA by DNA gyrase drives the decatenation of the sister chromatids by topoisomerase IV [8,56-58]. On bacterial plasmids even a small reduction in supercoiling on catenated plasmids drastically reduces the decatenation activity on the linked DNA circles [57]. On whole chromosomes, loss of the negative supercoiling introduced by gyrase into newly synthesized chromatids causes a dramatic loss of chromosomal partitioning after replication, even though gyrase does not directly decatenate linkages between the newly replicated chromosomes [20,59]. A crucial aspect of the resolution of SCI by supercoiling-mediated compaction is that it utilizes the power of ATP hydrolysis, by gyrase (or the condensin complex in eukaryotes – see below), to drive the resolution of any inter-chromosomal linkages. This ensures that the resolution reaction goes far beyond the expected point of equilibrium than would be anticipated without energy input.

Eukaryotic chromosome compaction

In eukaryotes there appears to be a more complex multilayered approach to intra-chromosomal compaction (historically referred to as chromosome condensation in eukaryotes). The first stage of intra-chromosomal compaction in eukaryotes occurs immediately following DNA replication with the wrapping of newly synthesized DNA into nucleosomes. Before passage of the replisome, DNA is unpackaged from nucleosomes. The repackaging of the newly synthesized DNA strands in the wake of the replisome would be predicted to put any catenated SCI generated by fork rotation under strain and thus promote their resolution by topoisomerase II (although this has not to date been experimentally tested). The next stage of compaction from the initial “string” of nucleosome packaged DNA – the so called 10 nanometre fibre – is thought to be through the binding of histone H1 to the nucleosomal fibre to generate higher order compaction. Interestingly, depletion of H1 from *Xenopus* egg extracts prevents the normal segregation of mitotic chromosomes in this system [60] consistent with unresolved DNA catenations persisting between the de-compacted sister chromatids. During mitosis the chromatin fibre is further condensed in a manner that correlates with widespread post-translational modifications of the unstructured histone N-terminal tails. In particular the phosphorylation of histone H3 at serine 10 (H3S10) is widely correlated with mitotic condensation [61,62]. Mutation of this residue to alanine in the ciliate *Tetrahymena* (but not in other systems [63]) demonstrated that this modification is important for the segregation of micronuclei, consistent with it being important for sister chromatid intertwine resolution [64].Whether this specific modification is generally directly or indirectly linked to compaction is not clear. However, a recent study has indicated that phosphorylation of H3S10 leads to the de-acetylation of the tail of H4, freeing it to interact with the surface of neighboring histones, thus driving further compaction [65].

In addition to the intra-chromosomal compaction provided by histone organization, it is also clear that the evolutionarily conserved condensin complex is required during mitosis to drive complete SCI resolution before chromosome segregation. Disruption of the condensin

complex in all eukaryotic organisms tested leads to a failure to fully segregate mitotic chromosomes and a defect in intra-chromosomal compaction [66]. Although the exact mechanism of condensin function is still actively debated, generally condensin appears to establish and maintain an ordered loop structure in mitotic chromosomes which drives self-compaction in a cell cycle dependent manner [67]. Detailed discussions of how condensin complexes could drive chromosomal compaction are provided elsewhere [68-70]. Briefly, one model of condensin action proposes that condensin complexes bring together two distant segments of the same chromosome, generating a mitotic loop [71]. This structural model of condensin is consistent with the preferred localization of condensin to the chromosome axis [72] where loops often appear to be anchored [73]. However, a wholly structural model of condensin appear to be at odds with the apparently dynamic interactions of some condensin complexes with chromatin [74] and that the effects of condensin action on intra-chromosomal structure are antagonized by topoisomerase II activity [75]. These observations would be consistent with the alternative model where condensin action leads to DNA supercoiling of the mitotic chromosome fibres. This model is derived from studies that have shown that condensin complexes drive the generation of positive supercoiling on plasmids *in vitro* following activation by mitotic kinases [76,77] and are required for mitotic supercoiling of plasmids *in vivo* in a manner that drives decatenation of sister chromatids [32]. Decatenation is presumably driven by the intra-chromosomal coiling exposing inter-chromosomal linkages to the outside of chromatid masses (Figure 5B) in a manner analogous to that proposed for supercoiling of chromatids in *E. coli* (Figure 5A). These data suggest that this strategy of driving the resolution of SCI has been conserved between prokaryotes and eukaryotes.

Related to the action of condensin during chromosome condensation, recent studies have highlighted the enigmatic role of the condensin related complex cohesin in chromosome compaction. *In vivo* cohesin's essential role appears to be the opposite of condensin, in that it is required to keep sister-chromatids together until anaphase [39]. However, it has been

known for some time that it is required for the initiation (but not maintenance) of mitotic rDNA compaction in budding yeast [78]. In *Xenopus* the dosage of cohesin complexes on chromosomes influences the final shape of the condensed chromosome, probably by antagonizing the activity of the condensin II complex [79] (metazoans have multiple condensin type complexes – reviewed by Hirano [66]). It has now being shown in mammalian cells that deletion of the cohesin regulator Wapl causes premature chromosome condensation in interphase and extensive chromosome bridging in anaphase [80]. Wapl is normally required for the pre-anaphase release of DNA bound cohesin complexes so its deletion leads to the stabilization of cohesin on DNA [81,82]. Therefore, it appears that stabilized cohesin promotes a disorganized type chromosome compaction that promotes sister-chromatid linkages rather than resolving them. At present it is not clear if these effects on mammalian chromosome are mediated through altering condensin function, or are cohesin specific effects on chromosome compaction.

The combination of intra-chromosomal compaction provided by chromatin organization and condensin action that occurs as eukaryotic cells passage through G2 to the metaphase/anaphase transition appears to be sufficient for the resolution of most SCI in cells. This is perhaps most clearly shown by direct observation of cytological linkages between segregating nuclei after treatment with topoisomerase II inhibitors at different stages of G2 and mitosis [83]. Inhibiting topoisomerase II activity in *Drosophila* cells during the early stages of mitosis leads to the formation of extensive chromosome bridges during anaphase whereas treatment after arresting in metaphase causes only modest changes in chromosome segregation [83]. This demonstrates the extent of SCI resolution that takes place before the metaphase to anaphase transition. However anaphase bridges are still observed after topoisomerase II inhibition at the metaphase to anaphase transition [11]. Therefore the final driver for resolving DNA catenation during anaphase is likely to be the force generated by the spindle apparatus when it pulls sister chromatids to opposite ends of the dividing cell [11,30]. The physical separation of previously linked DNA segments during

anaphase would clearly prevent re-catenation of the sister chromatids.

Sister chromatid intertwines generated during homologous recombination

In addition to the sister chromatid intertwines generated by fork rotation and DNA catenation or from unreplicated regions, there is another type of SCI that can be formed in cells. DNA damage can lead to double strand DNA breaks (DSBs) that are often repaired by homologous recombination. This requires strand invasion from one chromatid into the other generating a physical connection in the form of a double Holliday junction between the two sister-chromatids called a sister chromatid junction (SCJ) [84] (Figure 6). Generally SCJ formation is a result of DNA repair processes, where strand invasion from one sister chromatid to the other provides a template for error free repair [85]. In addition to SCJ formation following DSB processing and homologous recombination, SCJ can also be formed in the absence of DSBs. Hemi-catenane like recombination dependent SCJ, thought to be initiated from the pairing of the newly synthesized strands, are also formed behind the replication fork following DNA damage in S phase to facilitate repair mechanisms [86] (Figure 6). Like DNA catenations, all SCJ must be resolved before mitosis is completed or they would prevent normal chromosome segregation. Several pathways are available to resolve SCJ. The most favoured pathway appears to be the action of the type IA topoisomerase III working in conjunction with a RecQ type DNA helicase [87]. Potentially RecQ/topo III like complexes will resolve the two hetero-duplex regions between the junctions in a similar manner to that proposed for unreplicated DNA regions (Figure 2B). Genetically ablating Topoisomerase III results in a significant delay in mitosis and chromosome mis-segregation [88]. These defects in chromosomal segregation are suppressed by deletion of the RecQ helicase, Rqh1/Sgs1 [88,89]. This indicates that RecQ helicase action normally initiates the resolution of SCJ, and then Topo III must resolve the product of this action. In the absence of RecQ type helicase activity other pathways can

successfully resolve these linkages. From recent studies it has become clear that these support pathways are Holliday Junction specific nuclease complexes (reviewed by Matos and West [90]). The action of these complexes appears to be confined to mitosis by cell cycle kinase regulation, presumably minimising the possibility of abnormal nuclease action earlier in the cell cycle [90,91].

Although many of these HR resolving pathways are essential for viability only following treatment with exogenous DNA damaging agents which induce homologous recombination structures, genetic ablation of multiple, redundant SCJ resolution pathways often results in cell inviability in unchallenged cells [92]. This suggests that SCJ are formed in every cell cycle and that therefore at least one pathway of SCJ resolution must be present to allow normal chromosome segregation. This absolute requirement for SCJ resolution pathways could be due to high levels of endogenously derived DNA damage in cells or that the hemi-catenane like SCJ junctions are formed in every S phase to ensure faithful DNA replication [93].

Visualisation of SCI during anaphase

Increasing the frequency of formation, or disrupting the resolution, of sister chromatid intertwines in cells is generally associated with the appearance of anaphase bridges during mitosis. Inhibition of topoisomerase II, topoisomerase III or inhibition of DNA replication (preventing the normal resolution of single stranded parental DNA duplex intertwines) leads to a widespread increase in the number of anaphase bridges [11,12,30,83,94-96]. So anaphase bridges can be instigated by an excess of any of the SCI discussed in this review. The anaphase bridges detected to date can be classified as two distinct types. One is detectable with DNA intercalating dyes such as DAPI/Hoechst that appears to be chromatinized (i.e. histones localise to the DNA bridge), hence they are often referred to a chromatin bridges. The second type appears to be substantially de-chromatinized and is not

detectable with conventional DNA intercalating dyes. These “Ultra Fine Bridges” (UFB) are only observable either with labelled nucleotides or by immunolocalising a number of proteins potentially involved in the generation, sensing or resolution of the intertwined DNA in the DNA bridge [46,97]. These proteins include the PICH helicase, RecQ helicase, Topoisomerase III, RPA, Topoisomerase II and the repair scaffolding protein TopBp1 [12,13,46,97]. Both chromatid bridges and UFBs are induced by replication stress and loss of decatenation activity [11,13]. Therefore it is difficult to cytologically demonstrate if the bridging is due to unresolved DNA catenations, unreplicated regions or SCJ. Comparison of the localisation of mitotic chromosomal markers with UFBs have shown that UFBs in unperturbed cells frequently link centromeric DNA [46,97]. Since inhibition of topoisomerase II activity specifically causes a substantial increase in UFBs between segregating centromeric regions it seems likely that centromeric UFBs are generated from unresolved DNA catenation [46,97]. In contrast the inhibition of DNA replication, but not the inhibition of decatenation activity triggered UFB formation in the locality of known fragile sites on the arms of human chromosomes [12]. This argues that UFBs in this region are due to un-replicated single stranded SCI. At present, the reason why one type of bridge is formed instead of the other is unknown. Since all forms of SCI appear to lead to both types of bridge it has been proposed that it is the relative timing of the processing of the initiating structure, compared to other chromatin events e.g. chromosome compaction, that determines if an anaphase bridge becomes chromatinized or not [13].

SCI and cancer

Oncogene induced replicative stress is a common feature of cancers [98]. The replicative stress induced in these cells lead to DNA DSBs, elongation problems and an increase of chromosomal bridges in anaphase [99-101]. Recent studies have begun to address how common oncogenes cause replicative stress in cancer cells, including how oncogenes can alter replication by causing either too few or too many replication forks to fire during S phase

(reviewed by Hills and Diffley [102]). Both of these changes could lead to significant changes in the number of SCI in the cell. Too few origins being fired, for example following chronic Cyclin E overexpression [103,104], will lead to replication forks having to replicate far larger regions of DNA, increasing the risk of fork stalling while also decreasing the chance of a stalled fork being rescued by a neighbouring, converging fork. This will increase the frequency of unreplicated regions persisting into mitosis, increasing formation of single stranded SCI, particularly at known hard to replicate sites such as fragile sites [12]. Increased replication fork stalling will also lead to an increased chance of fork collapse and rescue by recombination-mediated processes, leading to an increase in SCJs. Conversely reduced origin usage will lower the number of replication termination events and therefore should reduce the formation of DNA catenanes in the cell. In contrast, overexpression of oncogenes such as HPV, Myc and Ras appears to lead to the firing of too many origins in S phase, and this also causes replicative stress [105-107] and potentially increases in SCI. The firing of excessive origins leads to the premature exhaustion of the dNTP pool [105,108] and of limiting replication factors [109], both of which lead to fork stalling and collapse, predicting an increase in single stranded SCI and SCJ as above. Excessive fork usage should also lead to an increase in the number of termination events in the cell and therefore an increase in DNA catenation.

As well as affecting the formation of SCI, the same mechanism that induce replication stress could also deregulate the mechanisms that resolve SCI. For example, overexpression of Cdc25A, which would be predicted to simulate the effects of oncogenic stress coupled with checkpoint deficiency, leads to both replication stress and inappropriate action of SCJ resolution mechanisms earlier in the cell cycle [110] . Therefore oncogenic stress could cause changes in mitotic SCI both through increasing the frequency of their formation during S phase and also through disruption of SCI resolution mechanism either before or during M phase.

Summary

It is clear that a small number of linkages between replicated chromosomes can persist following DNA replication in the form of the several types of sister chromatid intertwines described in this review. All the types of SCI are formed during DNA replication and repair and their frequency of formation can be influenced by numerous replication related factors. These include replication stress, DNA damage, replication through “difficult” chromosomal contexts, particularly those that may inhibit topoisomerase access ahead of the fork and the configuration of the replisome. In order to prevent these SCI from inhibiting chromosome segregation and causing aneuploidy, it is also clear that SCI resolution mechanisms are activated both before and during anaphase to resolve these structures. These mechanisms include intra-chromosomal compaction to drive the resolution of SCI by topoisomerases as well as the mitosis specific up-regulation of the activity of resolvase enzymes.

So at present we appear to have a reasonable understanding of how SCI can be produced and how cellular processes can resolve them. A substantial challenge for the future is to move beyond the general causes of SCI formation and their mechanisms of resolution to start understanding the specific context of the formation and resolution of each of the types of SCI. However, without SCI type specific markers, it is difficult to ascertain what types of SCI and at what frequency each are formed in both normal and cancer cells. Ideally, we would also have an assay to ascertain the genomic loci where SCI preferentially form in cells. Such assays could have important implications for cancer diagnostics and treatment. Detailed analysis of the SCIs formed in a specific cancer combined with knowledge of the genetic landscape of the cell could pinpoint the functionally relevant genomic instability pathways at work and allow interdiction of the exact processes maintaining viability in the chromosomally instable cell.

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Figure legends

Figure 1. Topoisomerase action in supercoil relaxation and DNA decatenation/catenation.

In a relaxed double-helical segment of DNA the strands are twisted around the axis once every 10.4-10.5 base pairs. In a relaxed closed circular molecule (A, middle) the addition or subtraction of twist (over and underwinding respectively) makes the molecule contort into a by coiling around itself in order to accommodate the change in winding while maintaining B-form, this is referred to as supercoiling. Overwinding leads to positive supercoiling (shown left of A and C). Underwinding leads to negative supercoiling (shown right of A and left of B). These over and underwinding stresses can be relaxed by topoisomerase action. (A) Type IB

enzymes such as Top1 relax the tension by transient nicking of one of the two strands, allowing them to rotate relative to one another. (B) Type IA enzymes such as topo III relax negative supercoiling/underwinding by catalysing a strand passage reaction on unwound regions. (C) Type II topoisomerases such as Top2 relaxes supercoiling tension by a double strand passage mechanism that effectively inverts the sign of the DNA crossing. (D) Catenation between circular DNA molecules connects them together like chain links. Completion of replication generates a final intermediate of catenated sister chromatids that cannot separate into the two daughter cells. Topoisomerase II can separate topologically linked duplex DNA molecules by use of its strand passage activity, introducing a transient double strand break in one DNA segment followed by the passage of an intact DNA molecule through the break before re-ligation in a process referred to as decatenation. Note that this process can also introduce catenated links into the previously unlinked DNA molecules.

Figure 2. Resolving the intertwines within duplex DNA.

(A) During elongation of DNA replication, (i) unwinding of the parental template separates the parental strands but does not resolve the intertwines that exist between the two strands. (ii) The intertwines between the strands are displaced into the region ahead of the fork leading to this becoming overwound, i.e. positively supercoiled (+). (iii) This tension is normally resolved by the action of either a type IB topoisomerase (such as eukaryotic topoisomerase I) or a type II topoisomerase (such as eukaryotic topoisomerase II), which act effectively as “swivelases” ahead of the fork to generate (iv) resolved and replicated sister chromatids. (v) However, Champoux and Been (see text) proposed a second mode of unwinding where the helical tension is relaxed by rotation of the fork to generate catenated DNA sister-chromatid intertwines behind the fork. Although these intertwines should not arrest forward elongation of replication, it is essential that the decatenating type II topoisomerases resolve all DNA catenation before the completion of cell division.
(B) On unreplicated DNA following bulk DNA replication. Replication stress is thought to lead

to fork collapse, particularly in hard to replicate regions such as fragile sites. If two converging forks break down this could lead to stretches of unreplicated DNA persisting after bulk DNA replication. Such regions are potential substrates for RecQ/topoIII type complexes, where the RecQ helicase generates sufficient local unwinding for the type IA topoisomerase topo III single strand passage reaction. The continued action of this complex on single stranded regions could resolve the remaining linkages between the two strands of duplex DNA.

Figure 3. Generation of DNA catenations at the termination of DNA replication

As replisomes converge, topoisomerases are sterically inhibited from relaxing the helical tension caught in the final few turns, triggering an increase in fork rotation to allow unwinding of the final few turns. The pre-catenane and catenated DNA SCI generated then have to be resolved by a type II topoisomerase.

Figure 4. Resistance to rotation is likely to increase with increasing complexity of the holo-replisome complex

In the case of the minimal replisome (top) the resistance to rotation of the elongating replisome is likely to be relatively low. Therefore for this replisome, a relatively high frequency of fork rotation and DNA catenation would be predicted to take place during DNA replication. However, the eukaryotic replisome holo-enzyme (bottom), has numerous non core proteins associated with it such as the replication fork protection complex, the Ctf4 trimer, the cohesion establishment machinery and histone deposition proteins. This is likely to be a much larger complex with a much greater resistance to rotation. In this case the replisome would be expected to rotate relatively infrequently and therefore DNA catenation would be relatively rare.

Figure 5. Model of how intra-chromosomal compaction drives complete resolution of SCI

(A) Supercoiling promotes decatenation. Monte Carlo simulation of singly linked catenanes. The red and yellow plasmids in the catenane in (i) are both relaxed. In (ii) the yellow DNA plasmid is supercoiled leading to its self-compaction and isolation of the catenated link. Reproduced from Postow et al. (2001) and reprinted with permission from Alexander Vologodskii and from Proceedings of the National Academy of Sciences of the USA.
(B) Condensin driven compaction drives decatenation. (i) Following DNA replication sister chromatids are connected by both DNA intertwines (highlighted by chequered ring) and cohesion complexes (pink elipses). Since the sister chromatid are relatively disorganized but closely linked by these connections, topoisomerase II cannot completely decatenate the sister chromatids. (ii) The ordered compaction introduced by condensin, illustrated here by a solenoidal coiling arrangement, will make inter-chromatid links accessible for removal by topoisomerase II during mitosis. (iii) In the absence of condensin, the compaction that takes place due to chromatin effects is likely to be disorganized and not drive inter-chromatid linkages to the outside of the chromatid masses limiting the ability of topoisomerase II action to resolve the SCI. This explains why, in the absence of condensin, the most obvious phenotype observed is defective chromosome segregation.

Figure 6. The structure of sister chromatid junctions (SCJs) and their pathways to resolution

Sister chromatid junctions occur either from the processing of double strands breaks to form double Holliday junctions (left) or from the formation of hemi-catenane like structures (right) formed to facilitate post replication repair pathways. These closely related structures could be formed from each other: The pairing of the unpaired strands of the hemi-catenane, through base pairing and type I topoisomerase intertwining will lead to a double Holliday junctions. Alternatively unwinding of one, of the two, paired heteroduplexes of the double Holliday junction by RecQ topo III activity would lead to formation of a hemi-catenane. Both structures can be unwound by RecQ helicases acting in combination with type 1A topo III

like topoisomerases, or cleaved by Holliday junction resolvases when they are activated specifically in mitosis.

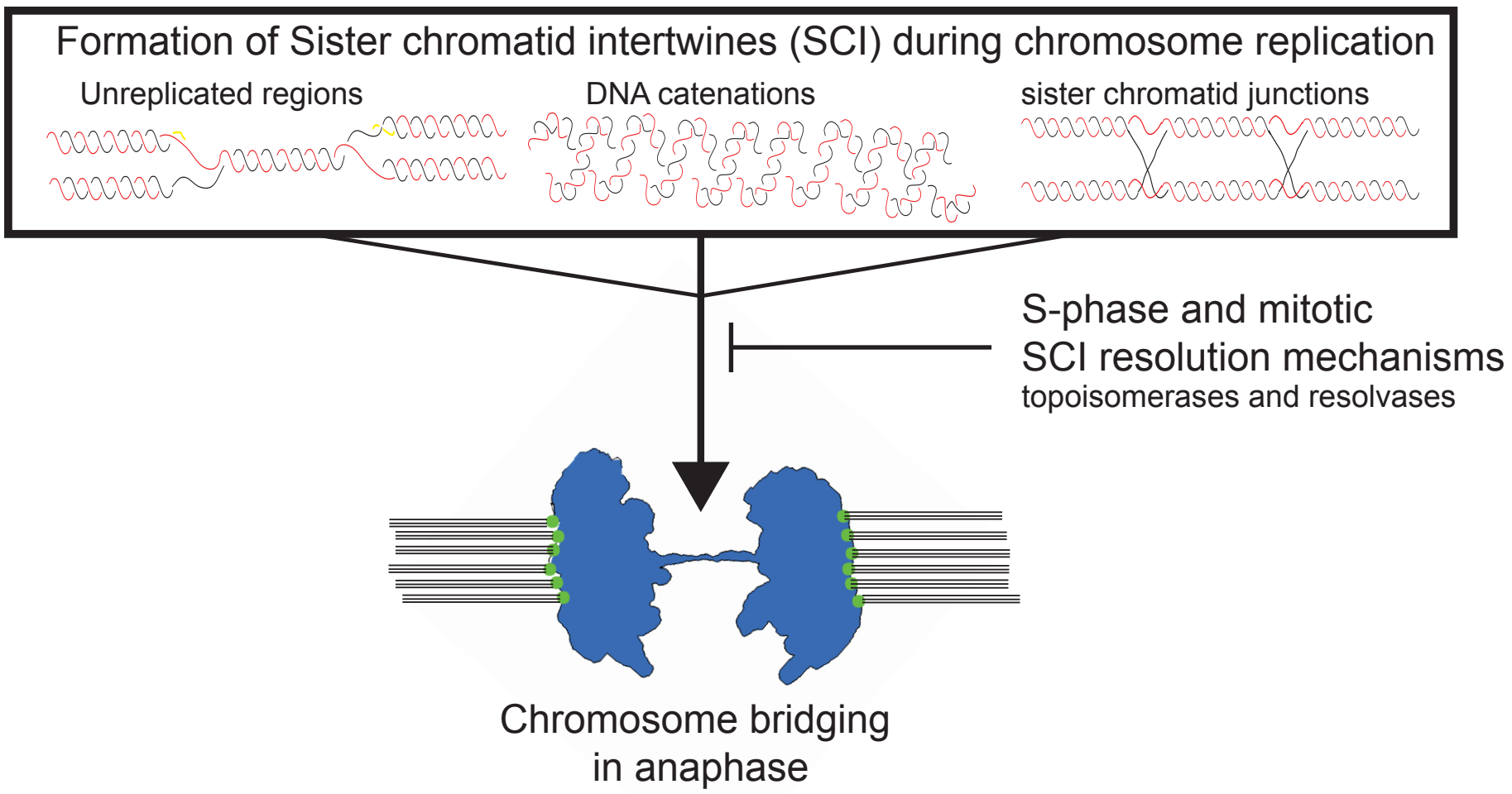
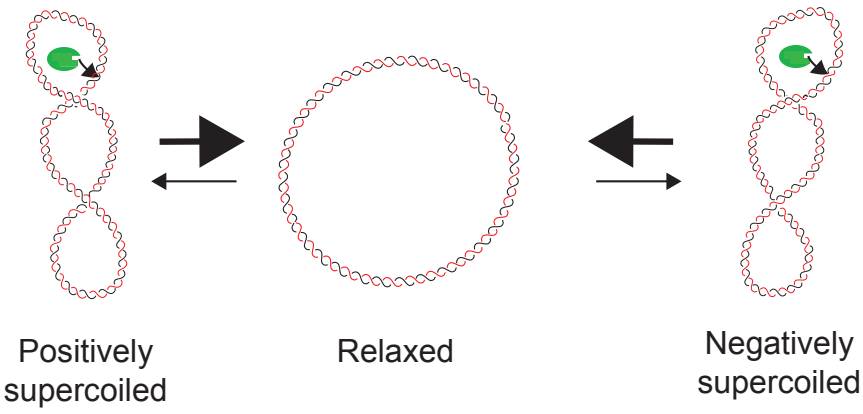

Formation of Sister chromatid intertwines (SCI) during chromosome replication
Unreplicated regions
DNA catenations
sister chromatid junctions
S-phase and mitotic
SCI resolution mechanisms
topoisomerases and resolvases
Chromosome bridging
in anaphase

Figure 1. Topoisomerase action in supercoil relaxation and DNA decatenation/catenation

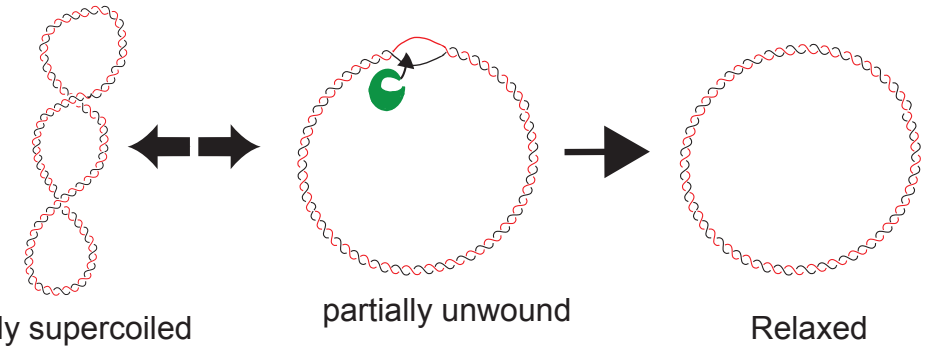
A

Type IB topoisomerase action
e.g. topo I/Top1
single strand breakage
and re-ligation



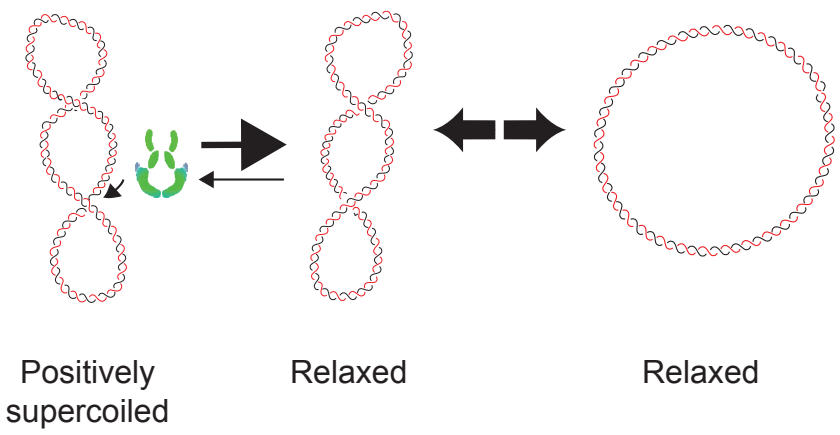
B

Type IA topoisomerase action
e.g. Top3/topoIII
single strand breakage
and strand passage



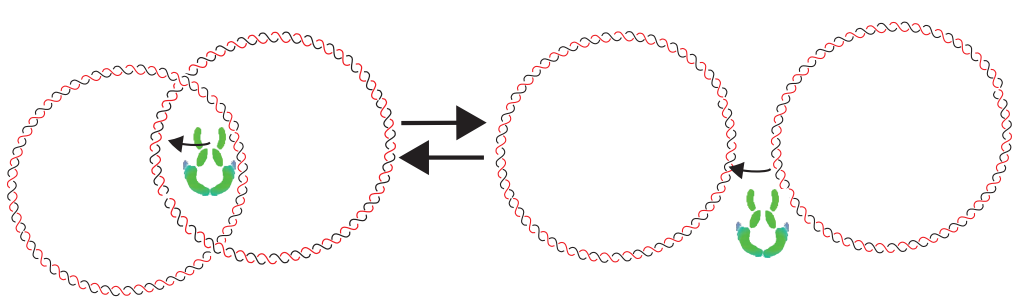
C

Type II topoisomerase
supercoil relaxation
e.g. topo II/Top2
double strand breakage
intra-molecular strand
passage and re-ligation



D

Type II topoisomerase
decatenation/catenation
e.g. topo II/Top2
double strand breakage
inter-molecular strand
passage and re-ligation



Type IB topoisomerase
e.g. topo I/Top1

Type IA topoisomerase
e.g. Top3/topoIII

Type II topoisomerase
e.g. topo II/Top2

Figure 2; Resolving the intertwines within duplex DNA

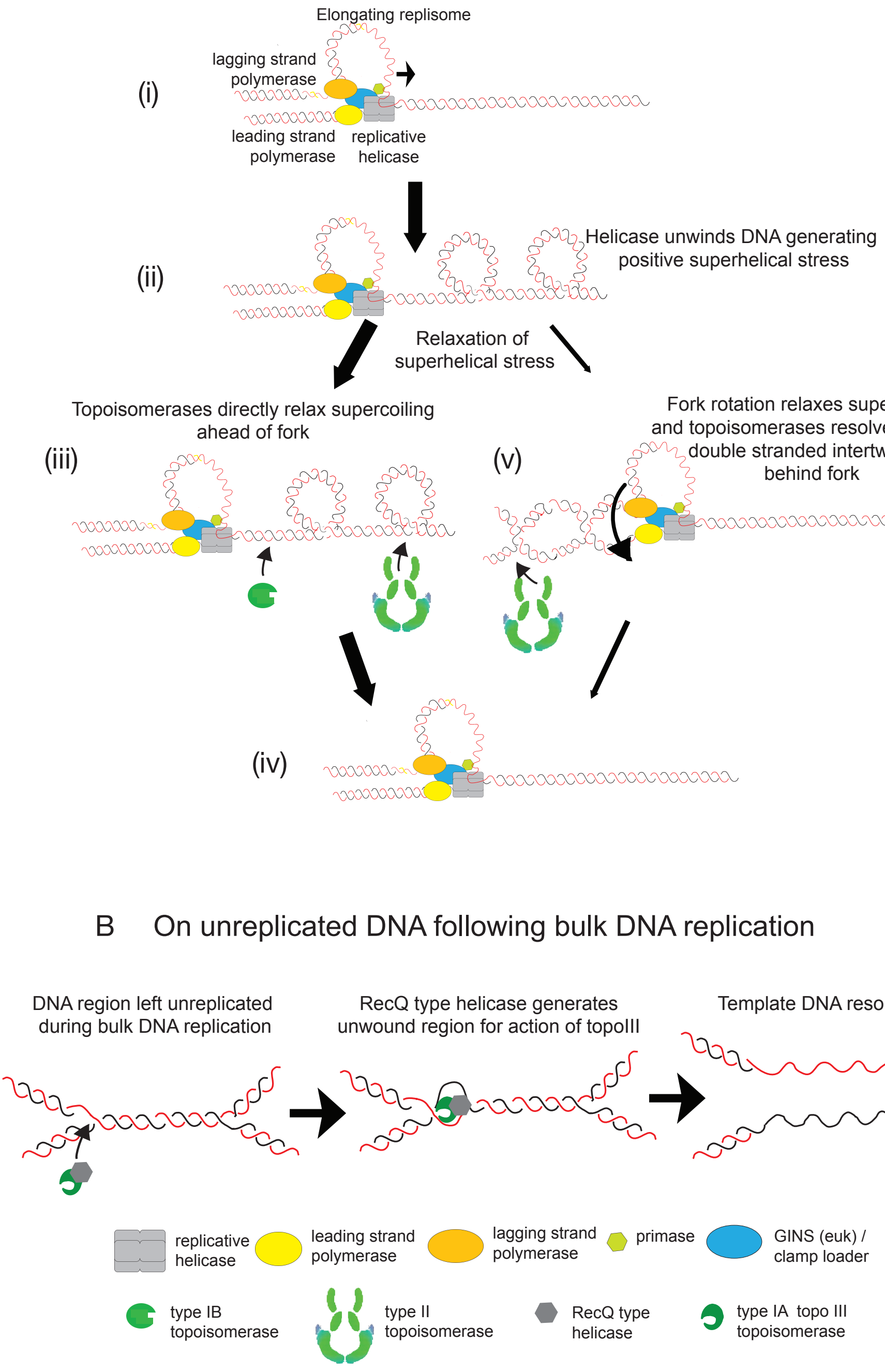


Figure 3. Generation of DNA catenations at the termination of DNA replication

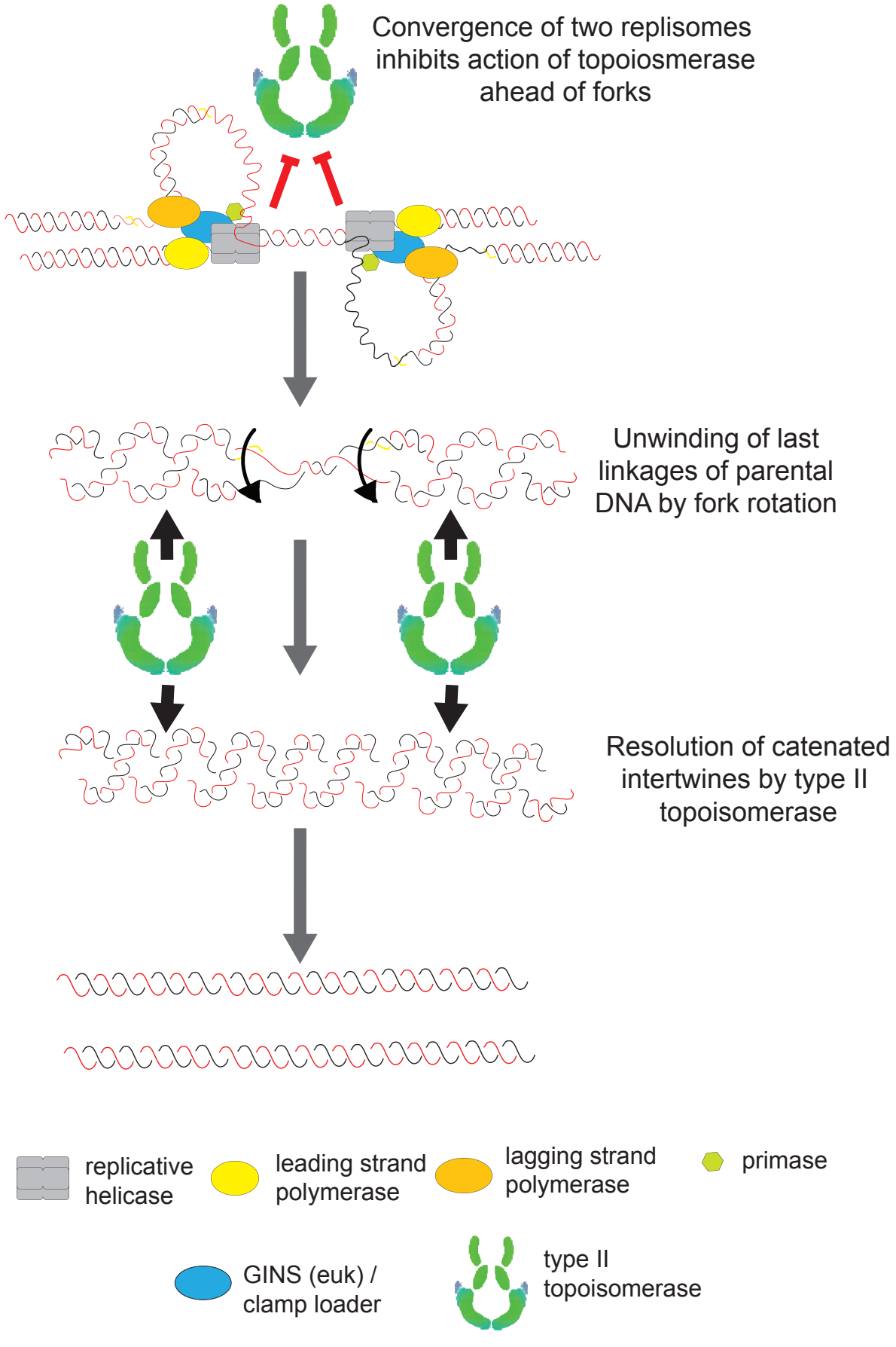


Figure 4; Resistance to rotation is likely to increase with increasing complexity of the holo-replisome complex

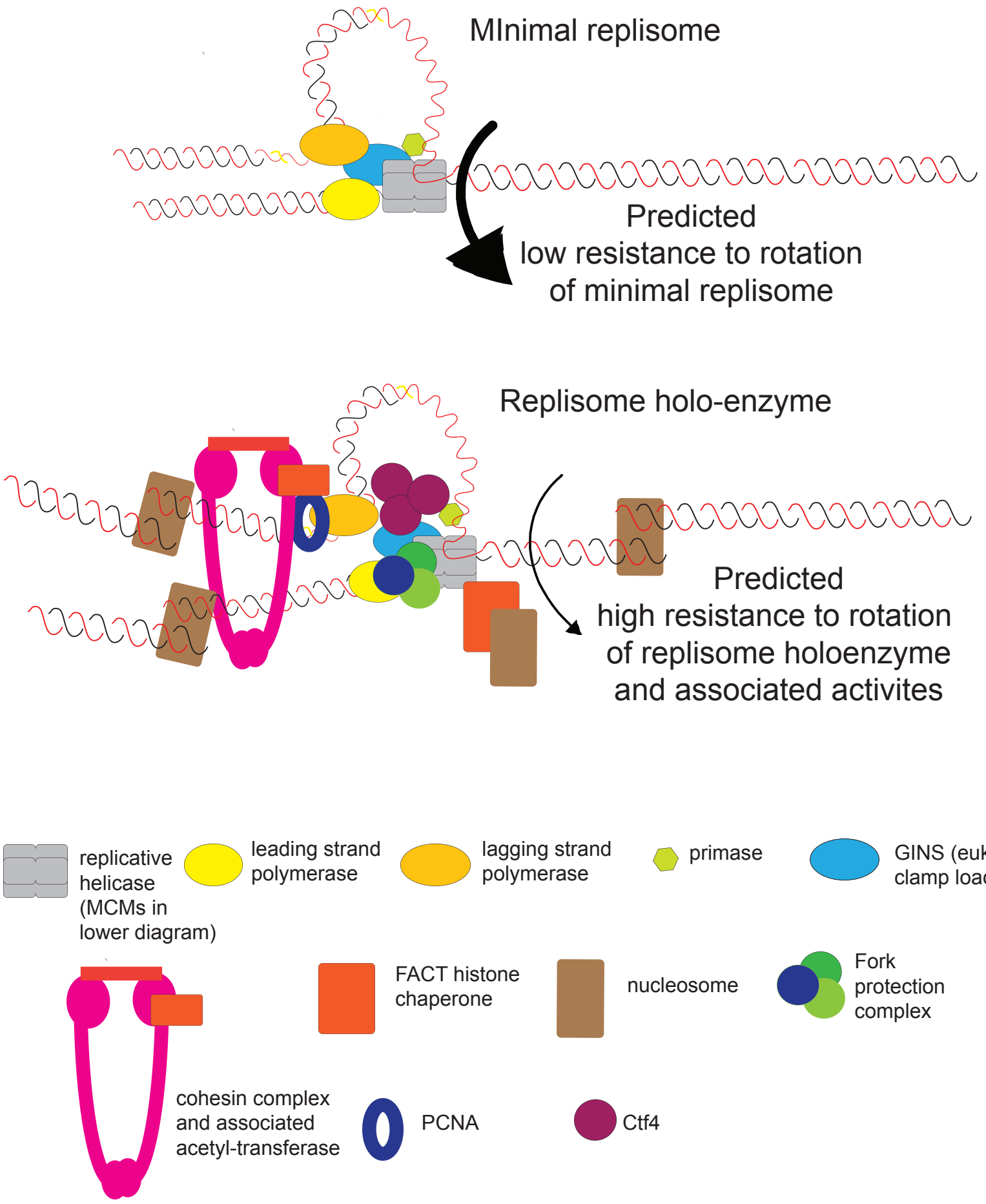


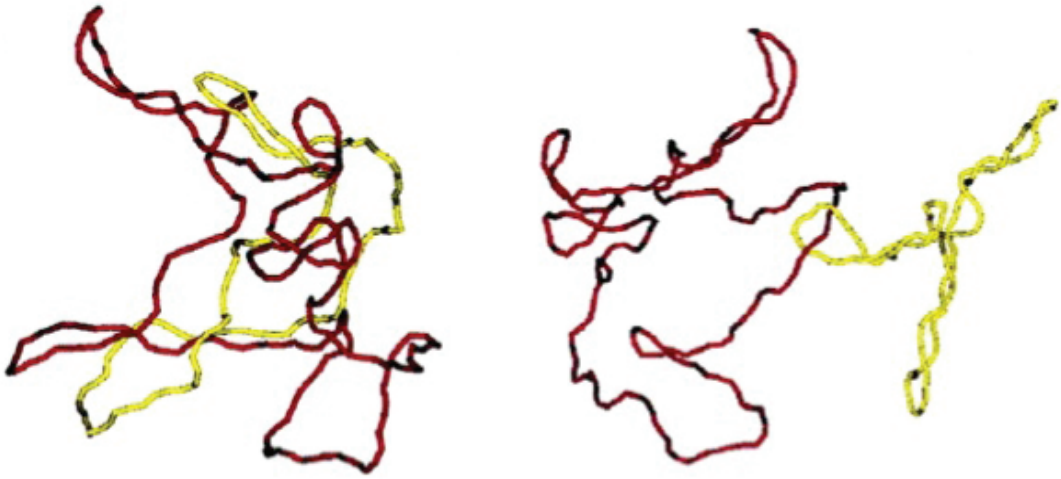
Figure 5. Model of how intra-chromosomal compaction drives complete resolution of SCI

A

Monte Carlo simulations of

(i) two relaxed plasmids linked by a single catenane

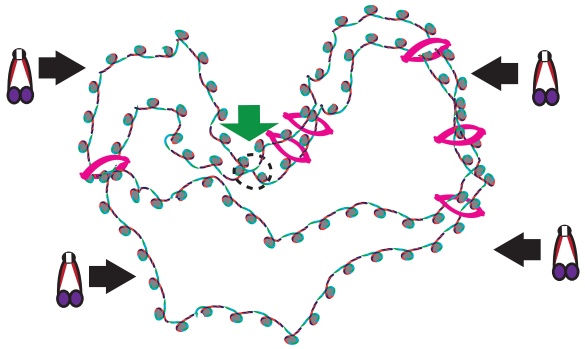
(ii) one relaxed and one supercoiled (and compacted) plasmid linked by a single catenane



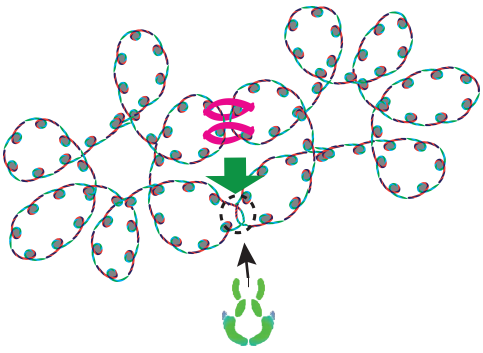
Postow L et al. PNAS 2001;98:8219-8226

B

(i) Pre-mitotic sister-chromatids

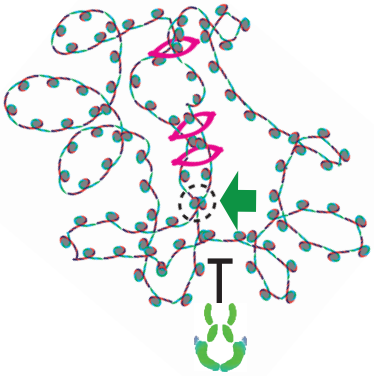


(ii) Wild type levels of condensin



Ordered compaction
Inter-chromosomal links accessible

(iii) Depleted levels of condensin



Poorly ordered compaction
Inter-chromosomal links inaccessible

topoisomerase II condensin complex cohesin complex

region highlighting location of DNA catenation

Figure 6. The structure of sister chromatid junctions (SCJs) and their pathways to resolution